Serotonin promotes acinar dedifferentiation following pancreatitis-induced regeneration in the adult pancreas

Saponara, Enrica ; Grabliauskaite, Kamile ; Bombardo, Marta ; Buzzi, Raphael ; Silva, Alberto B ; Malagola, Ermanno ; Tian, Yinghua ; Hehl, Adrian B ; Schraner, Elisabeth M ; Seleznik, Gitta M ; Zabel, Anja ; Reding, Theresia ; Sonda, Sabrina ; Graf, Rolf

Abstract: The exocrine pancreas exhibits a distinctive capacity for tissue regeneration and renewal following injury. This regenerative ability has important implications for a variety of disorders, including pancreatitis and pancreatic cancer, diseases associated with high morbidity and mortality. Thus, understanding its underlying mechanisms may help develop therapeutic interventions. Serotonin (5-hydroxytryptamine, 5-HT) has been recognized as a potent mitogen for a variety of cells and tissues. Here we investigated whether serotonin exerts a mitogenic effect in pancreatic acinar cells in three regenerative models, namely inflammatory tissue injury following pancreatitis, tissue loss following partial pancreatectomy and thyroid hormone-stimulated acinar proliferation. Genetic and pharmacological techniques were used to modulate serotonin levels in vivo. Acinar de-differentiation and cell cycle progression during the regenerative phase were investigated over the course of two weeks. By comparing acinar proliferation in the different murine models of regeneration, we found that serotonin did not affect clonal regeneration of mature acinar cells. Serotonin was however required for acinar de-differentiation following inflammation-mediated tissue injury. Specifically, lack of serotonin resulted in delayed up-regulation of progenitor genes, delayed formation of acinar-to-ductal metaplasia and defective acinar cell proliferation. We identified serotonin-dependent acinar secretion as a key step in the progenitor-based regeneration, as it promoted acinar cell de-differentiation and the recruitment of type 2 macrophages. Finally, we identified a regulatory Hes1-Ptfa axis in the uninjured adult pancreas activated by zymogen secretion. Our findings indicate that serotonin plays a critical role in the regeneration of the adult pancreas following pancreatitis by promoting de-differentiation of acinar cells.

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Enrica Saponara[1], Kamile Grabliauskaite[1#], Marta Bombardo[1#], Raphael Buzzi[1], Alberto B. Silva[1], Ermanno Malagola[1] Yinghua Tian[1], Adrian B. Hehl [2], Elisabeth M. Schraner[3], Gitta Seleznik[1], Anja Zabel[1], Theresia Reding[1], Sabrina Sonda[1*] and Rolf Graf[1*]

[1]Swiss Hepato-Pancreato-Biliary Center, Department of Visceral and Transplantation Surgery, University Hospital, Zurich; [2]Institute of Parasitology, University of Zurich; [3]Institutes of Veterinary Anatomy and Virology, University of Zurich, Switzerland

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# *Contributed equally*

* *Shared last authors*

Address correspondence to:

Pancreatitis Research Laboratory

Department of Visceral and Transplantation Surgery

University Hospital Zurich, Rämistrasse 100, 8091 Zurich, Switzerland

Tel.+41 44 255 33 55

Sabrina Sonda; sabrina.sonda@usz.ch

Rolf Graf; rolf.graf@usz.ch

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Abstract

The exocrine pancreas exhibits a distinctive capacity for tissue regeneration and renewal following injury. This regenerative ability has important implications for a variety of disorders, including pancreatitis and pancreatic cancer, diseases associated with high morbidity and mortality. Thus, understanding its underlying mechanisms may help develop therapeutic interventions. Serotonin has been recognized as a potent mitogen for a variety of cells and tissues. Here we investigated whether serotonin exerts a mitogenic effect in pancreatic acinar cells in three regenerative models, namely inflammatory tissue injury following pancreatitis, tissue loss following partial pancreatectomy and thyroid hormone-stimulated acinar proliferation. Genetic and pharmacological techniques were used to modulate serotonin levels *in vivo*. Acinar de-differentiation and cell cycle progression during the regenerative phase were investigated over the course of two weeks. By comparing acinar proliferation in the different murine models of regeneration, we found that serotonin did not affect clonal regeneration of mature acinar cells. However, it was required for acinar de-differentiation following inflammation-mediated tissue injury. Specifically, lack of serotonin resulted in delayed up-regulation of progenitor genes, delayed formation of acinar-to-ductal metaplasia and defective acinar cell proliferation. We identified serotonin-dependent acinar secretion as a key step in the progenitor-based regeneration, as it promoted acinar cell de-differentiation and the recruitment of type 2 macrophages. Finally, we identified a regulatory Hes1-Ptfa axis in the uninjured adult pancreas activated by zymogen secretion. Our findings indicate that serotonin plays a critical role in the regeneration of the adult pancreas following pancreatitis by promoting de-differentiation of acinar cells.

Keywords: serotonin; acinar secretion; de-differentiation; regeneration; ADM; pancreatitis; partial pancreatectomy.

Introduction

Despite a low homeostatic turnover, the adult exocrine pancreas has the ability to regenerate following an inflammatory insult. An increasing number of reports have revealed that during this regenerative process acinar cells, which constitute the most abundant cell population of the exocrine tissue, transiently de-differentiate and initiate a genetic program resembling the one found in embryonic pancreatic precursors (recently reviewed in [1,2]). Although numerous studies have identified crucial factors involved in the regeneration of acinar cells, the precise mechanisms initiating the cascade of events leading to their de-differentiation have not yet been identified. Serotonin (5-hydroxytryptamine, 5-HT), a monoamine stored and released by circulating platelets, has long been recognized as a potent bioactive molecule with mitogenic effects not only during embryonic development [3,4] but also in adult cells [5]. Examples of the mitogenic role of 5-HT in adult tissues include liver regeneration following hepatectomy [6], megakaryocyte colony formation [7], pulmonary artery smooth muscle cell proliferation [8] and beta cell expansion during pregnancy [9].

We recently showed that 5-HT plays a major role in the pathophysiology of the exocrine pancreas where it promotes acinar cell secretion and exacerbates the severity of inflammation at the onset of acute pancreatitis [10]. Here we investigated whether 5-HT acts as a mitogen for pancreatic acinar cells and thus promotes tissue regeneration. To this aim, we compared three types of regenerative stimuli, namely cerulein-induced pancreatitis, partial pancreatectomy and thyroid hormone supplementation, in mice

deficient in peripheral 5-HT (tryptophan hydroxylase 1 knocked-out, TPH1$^{-/-}$), and in wild type mice supplemented with 5-HT precursor.

Material and methods

Animal experiments

All animal experiments were performed in accordance with Swiss federal animal regulations and approved by the cantonal veterinary office of Zurich. Pancreatitis was induced in adult (8-10 weeks of age) wild-type (WT) C57BL/6 (Harlan Laboratories, Horst, The Nederlands) and TPH1$^{-/-}$ mice [11] on a C57BL/6 background (own breeding) via six intra peritoneal (i.p.) injections of 50 μg/kg cerulein administered hourly over a two week period, as described in Supplementary Materials and Methods and [12]. Pancreatectomy was performed according to [13], with the procedure adapted to remove 60% of the organ. 400 μg/kg 3,5,3-L-tri-iodothyronine (T3) was administered daily via i.p. injections for six days. 20 mg/kg 5-hydroxytryptophan (5-HTP) was administered by two subcutaneous injections per day, 12 hours apart. The treatment was started one day before cerulein treatment; control animals received 0.9% NaCl injections.

Histology, immunohistochemistry and immunoblotting

Detailed protocols and primary antibodies used in this study are listed in Supplementary Materials and Methods. Quantification of labelled cells was performed in at least 10 randomly selected high-power fields (×100) per slide. Non-acinar tissue areas (islets, vessels, fibrotic tissue) were excluded from the analysis.

Electron microscopy analysis

WT and TPH1$^{-/-}$ mice were harvested 8 hours after the beginning of cerulein treatment. Sliced pancreata were fixed in 2% glutaraldehyde/1% paraformaldehyde in 0.1M Na/K-phosphate for 90 min. After washing, the samples were postfixed with 1% osmium tetroxide in 0.1M Na/K-phosphate for one hour, dehydrated in a graded ethanol series, transferred to acetone for embedding in Epon and polymerized at 60°C for 2.5 days. Ultrathin sections were stained with uranyl acetate and lead citrate and examined at an acceleration voltage of 100 kV in a Philips CM 12 transmission electron microscope (Eindhoven, The Netherlands) equipped with a CCD camera (Ultrascan 1000, Gatan, Pleasanton, CA).

Transcript analysis

Total RNA was extracted from pancreata as previously described [14] and reverse transcribed with qScript™ cDNA SuperMix (Quanta Biosciences). Transcript levels were normalized using 18S rRNA as a reference and expressed as fold regulation relative to the value of untreated control animals. Taqman probes (Applied Biosystems) used in this study are listed in Supplementary Materials and Methods.

Results

Lack of 5-HT results in defective acinar cell proliferation and delayed ADM formation

To test whether 5-HT is mitogenic for acinar cells, we compared acinar proliferation following induction of pancreatitis in WT and in TPH1$^{-/-}$ mice, characterized by reduced 5-HT levels in the periphery but normal 5-HT signaling in the nervous system [10]. Unexpectedly, TPH1$^{-/-}$ animals showed a significantly higher number of acinar cells

positive for the general cell cycle activation marker Ki67 after one week of cerulein treatment compared with WT animals, while the number of bromodeoxyuridine (BrdU) positive cells in G1/S phase was similar in the two strains. Phospho-histone H3 (pH3) positive acinar cells in G2/M phase increased in TPH1$^{-/-}$ mice only 14 days after the beginning of the treatment (Fig. 1A, S1A). Acinar and interstitial cells were distinguished based on nuclear size, shape and location, as shown in Fig. S1B, C. Quantification of acinar cells in G2 phase, displaying punctuate pH3 staining, and in mitotic phase, with homogeneous nuclear pH3 staining [15], showed a reduced transition into mitotic phase in TPH1$^{-/-}$ animals one week after induction of pancreatitis (Fig. 1B).

In addition, formation of acinar-to-ductal metaplasia (ADM), a transient trans-differentiation observed during pancreatitis-induced regeneration, was delayed in TPH1$^{-/-}$ mice three days after induction of pancreatitis (Fig. 1C). The slower kinetics of ADM formation was not the result of defective expression or re-localization of β-catenin, a key a transcriptional activator promoting ADM formation [16]. β-catenin was similarly localized on the plasma membrane and nuclei of intact acinar cells in both strains (Fig. 1D) and was also found in ADM of WT animals and in the rare areas with reduced amylase content in TPH1$^{-/-}$ mice (Fig. 1E, arrowheads). Seven days after induction of pancreatitis, mature clustered ductal structures were visible in TPH1$^{-/-}$ mice and were similar to WT ADMs in terms of β-catenin expression, Sox9 up-regulation, amylase down-regulation and activation of αSma-positive stellate cells (Fig. 2A, S2). Taken together, these data indicate that, upon cerulein stimulation, TPH1$^{-/-}$ acinar cells are able to initiate the cell cycle; however, they display defective cell cycle progression and delayed ADM formation.

Lack of 5-HT delays acinar de-differentiation during pancreatitis

Proliferation of pancreatic acinar cells is preceded by a temporary de-differentiation and up-regulation of markers normally associated with pancreatic ductal or progenitor cells [17]. Concomitant with reduced ADM formation, TPH1$^{-/-}$ mice showed delayed up-regulation of the ductal marker cytokeratin 19 (CK19) three days after induction of pancreatitis (Fig. 2A, B). In addition, delayed up-regulation was also observed for several progenitor markers, including Sox9, Hes1, Notch1, Hnf1β. Ductal and progenitor gene expression was higher in TPH1$^{-/-}$ than in WT animals after 14 days of pancreatitis, with the exception of Aldh1, the expression of which was comparable in the two strains (Fig. 2A, B).

Noteworthy, delayed progenitor gene up-regulation in TPH1$^{-/-}$ mice was not accompanied by defective down-regulation of genes typical of the differentiated state, such as secretory granule enzymes and transcription factors controlling acinar cell identity, such as Mist-1[18-20] (Fig. S3). These data indicate that WT and TPH1$^{-/-}$ mice responded equally to the cerulein stimulus by down-regulating genes specific to mature differentiated acinar cells. However, amylase content did not decrease in TPH1$^{-/-}$ acini, as quantified by western blotting (Fig. 3A), enzymatic activity (Fig. 3B) and immunofluorescence analysis (Fig. 3C), indicating that down-regulation of zymogen transcripts was not sufficient to induce zymogen loss in these mice. Autophagy can reduce zymogen content by intracellular degradation [21]. However, autophagy was not differentially regulated in TPH1$^{-/-}$ mice, as shown by comparable processing of the autophagic protein LC3 (Fig. S4A). Similarly, autophagic marker p62 [22] showed

widespread expression in acini at 8 hours and down-regulation one day after induction of pancreatitis in both strains (Fig. S4B, C).

Increased zymogen secretion promotes acinar de-differentiation during acute pancreatitis

An alternative cellular mechanism explaining zymogen loss observed in acinar cells following pancreatitis could be the secretion of zymogens into extracellular space. We previously showed that TPH1$^{-/-}$ mice have impaired zymogen secretion [10]. This defective secretory process was further investigated at the ultrastructural level. Electron microscopy analysis revealed that zymogen granules were more tightly packed in TPH1$^{-/-}$ than in WT mice, both in control and after cerulein treatment (Fig. 3D). Furthermore, size distribution analysis showed that granule size, comparable in the two strains in the untreated controls, increased after stimulation of secretion, indicative of granule swelling preceding content expulsion [23]; however, the enlargement was higher in TPH1$^{-/-}$ (Fig. 3E), presumably a consequence of impaired secretion. Thus, our results show that reduced zymogen secretion observed in the absence of 5-HT is associated with defective zymogen loss followed by delayed up-regulation of progenitor genes. To test whether 5-HT-dependent zymogen secretion is required to initiate acinar de-differentiation following pancreatitis, we increased zymogen secretion 24 hours before cerulein administration by treatment with 5-hydroxytryptophan (5-HTP) (regimen scheme depicted in Fig. 4A), a precursor of serotonin that dose-dependently stimulates apical secretion of zymogens into pancreatic ducts (reviewed in [24]). This stimulated secretion is associated with a protective effect during induction of acute pancreatitis [25-27]. Under 5-HTP supplementation and one day of pancreatitis, we also observed lower

levels of inflammatory infiltrates in the pancreas (Fig. 4B), chemokine expression (Fig. 4C) and serum amylase (Fig. 4D), likely due to the increased 5-HTP-stimulated apical secretion counteracting the aberrant baso-lateral secretion induced by cerulein. The reduced severity of the disease did not translate in reduced acinar cell de-differentiation, as 5-HTP+cerulein-treated mice showed robust amylase loss in the pancreas similar to cerulein-treated mice (Fig. 4E). In addition, these mice had increased RNA levels of progenitor gene Hes-1 (Fig. 4F) and earlier expression of Sox-9 in acinar cell nuclei (Fig. 4G). These results suggest that stimulation of zymogen secretion enhances acinar de-differentiation following induction of pancreatitis. However, despite reducing the initial inflammation, one set of 5-HTP supplementation (regimen scheme depicted in Fig. S5A) was not sufficient to promote pancreatic regeneration, as shown by comparable levels of acinar and interstitial cell replication (Fig. S5B) and inflammatory cell infiltration (Fig. S5C) three days after induction of pancreatitis. In addition, increasing the dose of 5-HTP supplementation stimulated the expression of progenitor (Fig. S5D) and inflammatory genes (Fig. S5E) compared to one set of 5-HTP. 5-HTP supplementation over 14 days further boosted the up-regulation of inflammatory genes (Fig. S5F), and resulted in an increased trend of acinar cell replication (Fig. S5G), ADM formation (Fig. S5H) and inflammatory infiltration (Fig. S5I), suggesting that supplementation with exogenous 5-HTP did not ameliorate disease progression.

Lack of 5-HT delays the expression of MMPs during pancreatitis

As inflammatory cells play an active role in ADM formation, we then tested the level of pancreatic inflammation in the two strains. TPH1$^{-/-}$ mice showed reduced inflammation after one [10] and three days of pancreatitis (Fig. 4H, S6A). At this time point,

expression of type-2 macrophage markers Mgl1 and Mrc1 was also reduced in TPH1$^{-/-}$ mice, while the type-1 marker Nos2 was comparable in the two strains (Fig. 4I), Macrophage marker expression increased in TPH1$^{-/-}$ mice at later time points (Fig. S6B). Cytokines secreted by macrophages stimulate matrix metalloproteinase (MMP) synthesis and secretion in acinar cells, thus driving digestion of extracellular matrix and ADM formation [28]. Concomitant with reduced macrophage infiltration, TPH1$^{-/-}$ mice showed a reduced trend of MMP7 (Fig. 4L) and TNFα expression (Fig. S6C).

Then we digested the pancreatic extracellular matrix with collagenase and compared de-differentiation of isolated acini after culture in suspension for 24 hours, as described [29]. In this *in vitro* setting, de-differentiation was similar in WT and TPH1$^{-/-}$ acini, with or without exogenous 5-HT added to the medium (Fig. 4M), suggesting that the 5-HT mediated effect is exerted up-stream of extracellular matrix digestion.

Analysis of vascular associated factors, including CD146 and VEGF, did not show alterations in the two strains after three days of pancreatitis, when the major ADM difference was observed, but their expression remained elevated in TPH1$^{-/-}$ mice after 14 days of the disease (Fig. S6D).

Lack of 5-HT does not alter clonal regeneration of acinar cells following partial pancreatectomy and T3 administration.

As we found that 5-HT supports progenitor-based regeneration following pancreatitis, we then investigated whether zymogen secretion is also required for pancreatic regeneration that does not depend on robust acinar de-differentiation and activation of progenitor genes. To this aim, we analyzed acinar replication following 60% partial pancreatectomy (PPX) that triggers a regenerative response preferentially based on

clonal division of differentiated acinar cells. Acinar replication, albeit lower than the one induced after pancreatitis at all the time points analyzed, peaked one week following PPX (Fig. 5A). Importantly, TPH1$^{-/-}$ mice did not show accumulation of Ki67 positive acinar cells (Fig. 5A, B). The replication peak was not preceded by robust expression of progenitor genes, the up-regulation of which was lower than during pancreatitis and comparable in the two strains, with the exception of Hes1 that increased at the two week time point only in WT mice (Fig. 5C). In addition, pancreata isolated after pancreatic resection showed comparable amylase content (Fig. 5D) and minimal levels of inflammation, apoptosis and fibrosis in the two strains (Fig.S7A-C). To test the effect of 5-HT in another model of inflammation-independent regeneration, we induced acinar proliferation by administering the thyroid hormone 3,5,3-L-tri-iodothyronine (T3) [30,31]. T3 treated WT and TPH1$^{-/-}$ mice showed similar levels of acinar replication (Fig. 5E) and minimal leukocyte infiltration (Fig. 5F). Collectively, these data revealed that acinar replication following PPX and T3 administration is not affected by the lack of 5-HT.

5-HT receptors are up-regulated during pancreatic regeneration

As 5-HT signals via specific 5-HT receptors and transporter, we then compared their expression during pancreatic regeneration. Type 1 and 2 receptors, known to play a role in regeneration of liver and heart [6,32,33] and found up-regulated in pancreatic cancer [34], were up-regulated following pancreatitis in WT mice and showed a delayed kinetic in TPH1$^{-/-}$ animals (Fig. S8A). Analysis of samples with reduced inflammation, such as TPH1$^{-/-}$ and 5-HTP supplemented WT mice one day after pancreatitis, indicated that inflammatory cells substantially contribute to the observed level of receptor expression, except in the case of 5-HT2A (Fig. S8B). Similarly, PPX samples, characterized by

minimal tissue inflammation, showed lower 5-HT receptor and transporter expression compared with pancreatitis specimens (Fig. S8C). Interestingly, expression of 5-HT2B robustly increased in WT mice 14 days after PPX.

To test whether acinar cells also express 5-HT receptors and transporter, we isolated pancreatic acini one day after pancreatitis induction. In WT acinar cells, expression of 5-HT receptors, but not transporter, increased upon disease induction. TPH1$^{-/-}$ acini had an opposite trend of receptor expression compared with WT cells, showing lower type 1 and higher type 2 receptor levels (Fig. S8D). Collectively, these results indicate that pancreatic regeneration is associated with up-regulation of 5-HT receptors and transporter, which can be expressed not only by infiltrating cells but also by pancreatic acini.

Hes1-Ptf1a axis is activated in the uninjured adult pancreas

As we observed that 5-HTP treatment enhanced acinar de-differentiation following induction of pancreatitis (Fig. 4E, F), we then tested whether this regulation was observed also in the absence of tissue damage. 5-HTP-treated WT mice had normal levels of serum and tissue amylase (Fig. 4C, D) and no Sox9 expression in acinar cells (Fig. 6A), suggesting that increased secretion into ducts is not sufficient to activate acinar de-differentiation. However, 5-HTP treatment increased the expression of the progenitor gene Hes1 in the pancreas (Fig. 6B), albeit at a lower level than following 5-HTP+cerulein treatment. Hes1 is a critical orchestrator of pancreatic embryogenesis (reviewed in [35]), as it regulates maintenance of progenitor cells and timing of cell differentiation by antagonizing the function of Ptf1a, a basic helix-loop-helix transcription factor that promotes zymogen synthesis [36]. Hes1 up-regulation following 5-HTP

treatment was concomitant with Ptf1a down-regulation (Fig. 6B). Moreover, early time points following cerulein treatment showed initial Ptf1a up-regulation followed by Hes1 expression and subsequent Pft1a down-regulation. 24 hours after induction of pancreatitis, Ptf1a levels were higher in TPH1$^{-/-}$ mice with delayed Hes1 up-regulation (Fig. 6C). This suggests that the inhibitory function of Hes1 on Ptf1a expression described during development may be active also in the adult pancreas. We further explored the Hes1-Ptf1a regulation in an *in vitro* system where the acinar cell line AR42J was stimulated toward a more differentiated and secretory phenotype by treatment with dexamethasone [37]. 48 hours of dexamethasone treatment changed the cell morphology from a more spheroid to flattened shape (Fig. S9A), led to decreased replication of AR42J cells (Fig. S9B), increased amylase content (Fig. 6D) and decreased Sox-9 expression (Fig. 6E). Similar to what we observed *in vivo*, AR42J differentiation showed an initial increase of Ptf1a expression that was then reduced following Hes1 up-regulation (Fig. 6F).

Taken together, the *in vivo* and *in vitro* results presented here show that conditions of increased zymogen secretion and synthesis in acinar cells are associated with the activation of the Hes1-Ptf1a axis. These data not only indicate that different progenitor genes are regulated in a different manner in acinar cells, but also that the Hes1-Ptf1a axis may contribute to maintain physiological zymogen levels in the adult pancreas in the absence of acinar cell damage.

Discussion

The remarkable regenerative capacity observed in organs of several non-mammalian vertebrates often involves the de-differentiation of mature cells (reviewed in [38]).

Notably, cell de-differentiation is also observed in the regeneration of the adult exocrine pancreas of mammals following pancreatitis. In this organ, differentiated acinar cells transiently revert into a progenitor state following injury, thus likely acting as facultative progenitor cells and initiating a replicative program leading to exocrine tissue repair (reviewed in [1,39,40]). This de-differentiation of adult pancreatic acinar cells is a critical process within the regenerative mechanism, as *bona fide* resident stem cells have not been reported in the adult pancreas (reviewed in [39]). Our results showed that, differently from what has been observed in other cell types and tissues [6,33,41-46], 5-HT does not act as a strong mitogen for acinar cells, as acinar replication was unchanged *in vivo* in the absence of 5-HT following 60% pancreatectomy and T3 stimulation and *in vitro* in AR42J acinar cells treated with 5-HT (data not shown). However, 5-HT is required for acinar de-differentiation following inflammatory-mediated tissue damage. In this context, it is important to mention that 60% pancreatectomy is characterized by a moderate level of acinar cell de-differentiation and replication compared with the more robust regeneration observed during induction of pancreatitis. These observations support the notion that, according to the type and extent of tissue injury, the mammalian pancreas has the ability to trigger different regenerative mechanisms, which are driven by activation of a progenitor-like program or by a simple, clonal proliferation of differentiated acinar cells (reviewed in [39,47]). Furthermore, as progenitor-based and clonal modalities of regeneration are not mutually exclusive and are likely to co-exist, the clonal regeneration independent from zymogen secretion is likely to account for the replication observed in TPH1$^{-/-}$ mice with compromised progenitor-based regeneration.

A major challenge undertaken in our study was to elucidate the mechanisms by which 5-HT affects acinar de-differentiation and ADM formation. We identified 5-HT-dependent secretion of zymogen content as one of the step promoting up-regulation of progenitor-like genes, suggesting that physical removal of zymogens via secretion has to occur to allow de-differentiation of adult acinar cells. However, additional mechanisms are likely to contribute to the observed delayed de-differentiation and ADM formation in the absence of 5-HT. We found that reduced secretion in TPH1$^{-/-}$ mice limits chemokine production and leukocyte infiltration in the early stages of cerulein-induced pancreatitis ([10] and this work). Thus, it is possible that the few infiltrating leukocytes released a limited amount of pro-inflammatory cytokines that may not have been sufficient to stimulate acinar de-differentiation and ADM formation. Indeed, TPH1$^{-/-}$ mice had reduced levels of TNFα and MMP, both factors critical to induce ADM formation [28]. The comparable de-differentiation observed *in vivo* in isolated acini supports the hypothesis that the 5-HT effect is not acinar cell autonomous but is mediated by macrophage-stimulated extracellular matrix digestion. However, it has to be noted that, despite sharing phenotypic similarities, the *in vitro* acinar de-differentiation may not completely reflect the molecular mechanisms occurring in the *in vivo* de-differentiation upon pancreatitis induction. Indeed, the process of acinar isolation *per se* activates Ras and its down-stream effectors ERK and AKT [29], the activity of which promotes cell de-differentiation. Thus, it is possible that the *in vitro* procedure bypasses a cell autonomous, 5-HT-mediated process occurring *in vivo*. In support of this hypothesis, 5-HT has been reported to act upstream Ras-dependent ERK1/2 activation via binding to different 5-HT receptors [48-50].

In this regard, an alternative explanation for the aberrant progenitor-based regeneration observed in TPH1$^{-/-}$ mice is that 5-HT directly stimulates the expression of progenitor genes via 5-HT receptor signaling or intracellular mechanisms following its uptake. We found that type 1 and 2 5-HT receptors were up-regulated during pancreatic regeneration, following both pancreatitis and PPX. Of note, type-1 5-HT receptors were recently described to promote proliferation of human pancreatic cancer cells [34]. Finally, we also discovered an activation of the Hes1-Ptf1a axis in the uninjured pancreas. Both under *in vivo* and *in vitro* experimental conditions, Hes1 up-regulation was accompanied by down-regulation of Ptf1a expression. This suggests the presence of a tightly regulated control of Ptf1 expression by Hes1 in the adult pancreas, reminiscent of the embryonic situation. Hence, our data are in support of a dual role of Hes1 in the pathophysiology of the pancreas. In physiological situations, increased zymogen secretion is associated with moderate Hes1 up-regulation, which limits Ptf1a expression and thus contributes to acinar homeostasis by preventing excessive zymogen expression. In pathological situations that trigger acinar replication, a higher Hes1 up-regulation occurs with a consequent reduction of transcription factors and proteins required to maintain acinar function. This down-regulation of differentiation genes may provide the cells with the suitable environment to up-regulate a broader set of progenitor genes, leading to a robust acinar proliferation and organ repair. Collectively, these results indicate that expression of progenitor genes is not a synchronous event. Therefore, similarly to what was observed in the determination of diverse cell fates during development [51], the different time of expression of these transcription factors in the adult pancreas may reflect their distinctive function during homeostasis and regeneration.

While highlighting the impact that 5-HT exerts on the de-differentiation and proliferation abilities of pancreatic cells under different regenerative conditions, this study nevertheless has some limitations. First, the use of a general TPH1 knocked out model system precludes the dissection of the individual cell contribution in the synthesis of 5-HT. Serotonin is mainly produced by intestinal enterochromaffin cells and stored and released by circulating platelets. However, additional sources of serotonin exist, including nerve fibers, which are abundant in the pancreatic tissue, β-cells [52] and even acinar cells [53]. In addition, further studies are necessary to characterize the role of the different 5-HT receptors present on acinar and non-acinar cells to fully understand their contribution during pancreatic regeneration. Finally, an aspect of the 5-HT biology that has not been addressed in this study is a thorough characterization of vascular function. Disturbances in microcirculation, including vasoconstriction, are associated with acute pancreatitis (reviewed in [54]). As 5-HT is a potent vasoconstrictor, it will be worth exploring whether alterations in the vascular biology contribute to the observed phenotype in $TPH1^{-/-}$ mice.

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Contributors The authors of this manuscript contributed in the study design, acquisition, analysis, interpretation of data, drafting and critical revision of the manuscript. ES, KG, MB, RB, ABS, EM, GS performed experiments, generated and analyzed data; ABH

performed confocal microscopy, generated and analyzed data; EMS performed electron microscopy; YT performed partial pancreatectomy; AZ performed experiments and generated data; TR generated transgenic lines; RG, SS designed the study; ES, SS wrote the manuscript; RG, ABS revised the manuscript for content, analysis and interpretation of data. All authors approved the submitted version.

List of abbreviations ADM, acinar-to-ductal metaplasia; TPH1$^{-/-}$, tryptophan hydroxylase 1 knocked-out; PPX, 60% partial pancreatectomy; 5-HTP, 5-hydroxytryptophan; T3, 3,5,3-L-tri-iodothyronine.

Data sharing statement: no additional data

List of online supporting material:

Supplementary material and methods and four supplementary figures

Figure legends

Figure 1. Lack of 5-HT results in defective acinar cell proliferation and delayed ADM formation. **A**. Quantification of replication markers expressed in pancreatic acinar cells showed increased number of Ki67 positive cells in TPH1$^{-/-}$ mice after 7 and 14 days of pancreatitis. **B**. Relative amount of acinar cells in G2 and M phases determined by pH3 staining pattern 7 and 14 days after pancreatitis induction. Data are expressed as percentage of the total number of pH3 positive cells with punctate (G2 phase) or complete nuclear staining (M phase). **C**. Hematoxylin-Eosin (H&E)-staining and ADM quantification showed lower amount of ADM (asterisks) in TPH1$^{-/-}$ mice after three days of pancreatitis induction. Lower panels: enlarged views of insets three days after pancreatitis. Note the presence of mature ADM in WT mice characterized by loss of acinar content and tubular complex formation. TPH1$^{-/-}$ pancreata presented sporadic de-

regulation of acinar content (stars) without re-organization into tubular structures. **D**. Confocal pictures showing nuclear localization of β-catenin in WT and TPH1$^{-/-}$ pancreata three days after induction of pancreatitis. Nuclei are stained with DAPI (blue). **E**. Amylase and β-catenin staining three days after induction of pancreatitis. Circled area and asterisk in WT pancreata represent ADM. Right panels: enlarged views of insets. Arrowhead, area with reduced amylase content and increased β-catenin expression in TPH1$^{-/-}$ pancreata. Results are average ± SEM (n=5), *p<0.05. Scale bars: 50 μm.

Figure 2. Lack of 5-HT delays acinar de-differentiation during pancreatitis. **A**. Cytokeratin 19 (CK19), Sox9, β-catenin and amylase staining three and seven days after induction of pancreatitis. Nuclei in the IF pictures are stained with DAPI (blue). **B**. qPCR of ductal and progenitor markers during the indicated times of cerulein-induced pancreatitis showed delayed expression in TPH1$^{-/-}$ pancreata. Results are average ± SEM (n=5), *p<0.05. Scale bars: 50 μm.

Figure 3. Lack of 5-HT prevents loss of amylase during pancreatitis. Biochemical determination of amylase activity (**A**) and immunoblotting of 10 μg of proteins (**B**) showed down-regulation of amylase in pancreatic tissue of WT but not TPH1$^{-/-}$ mice one day after cerulein treatment. **C**. Immunostaining of amylase during 14 days of pancreatitis induction showed decreased protein expression in WT but not in TPH1$^{-/-}$ mice one day after cerulein treatment. **D**. Electron micrographs of WT and TPH1$^{-/-}$ pancreata. Note the close proximity of zymogen granules with reduced inter-granule space in TPH1$^{-/-}$ mice, both in control and cerulein treatment. L, acinar lumen with evident microvilli. V, zymogen granule, M, mitochondrion. RER, rough endoplasmic reticulum. Right panel, quantification of the area occupied by granules expressed as

percentage of total area. **E**. Size distribution of zymogen vesicle diameter. Note the higher abundance of vesicles with large diameter in TPH1$^{-/-}$ mice following pancreatitis induction. Results are average ± SEM (n=5), *p<0.05. Scale bars: 1 μm.

Figure 4. **A**. Scheme depicting the combined regimen with 5-HTP and cerulein (Cer). 0.9% NaCl was used as vehicle control. Circles indicate days of treatment starting from the first cerulein injection and triangles indicate days of animal harvest. Pan-leukocyte immunostaining with anti-coronin 1 (**B**) and qPCR of pro-inflammatory chemokine MCP-1 (**C**) showed lower inflammation upon 5-HTP supplementation one day after induction of pancreatitis. Serum (**D**) and tissue (**E**) levels of amylase 8 hours after pancreatitis induction following 5-HTP supplementation. qPCR of Hes1 (**F**) and immunostaining of Sox9 (**G**) one day after pancreatitis induction following 5-HTP supplementation. **H**. inflammatory cell quantification after PU.1 immunostaining showed reduced infiltration in TPH1$^{-/-}$ mice three days after pancreatitis induction. **I**. qPCR of type1 and 2 macrophage markers three days after pancreatitis induction. **L**. qPCR of matrix metalloproteinase 7 (MMP7) at the indicated days of pancreatitis induction. **M**. qPCR of progenitor and zymogen markers in isolated acini (inset) cultured for 24 hours in suspension to induce differentiation. 5-HT was added to the medium at 20 μM concentration. Results are average ± SEM (n=5), *p<0.05. Scale bars: 50 μm.

Figure 5. Lack of 5-HT does not alter clonal regeneration of acinar cells following 60% pancreatectomy (PPX) and thyroid hormone T3 stimulation. **A**. Quantification of replication markers expressed in pancreatic acinar cells at the indicated times after partial pancreatectomy. **B**. Immunostaining of Ki67 at the indicated time points following PPX. Pictures represent an inset (1:10) of the original images used for Ki67

quantification. **C**. qPCR of progenitor-cell markers at the indicated times after PPX. Note that WT but not TPH1$^{-/-}$ mice increased Hes1 expression 14 days after surgery. Expression levels obtained in WT mice after cerulein induced pancreatitis (WT CIP) are shown as a comparison. **D**. Immunostaining of amylase showed comparable protein contentn in WT and TPH1$^{-/-}$ mice following PPX. **E**. Quantification of Ki67 expressed in pancreatic acinar cells following T3 administration for six days. **F**. Pan-leukocyte immunostaining with anti-coronin1 showed comparable level of inflammatory cells in control and treated mice. Results are average ± SEM (n=3-5), *p<0.05. Scale bars: 50 μm.

Figure 6. Increased zymogen secretion stimulates Hes1 expression in the uninjured pancreas. **A**. Left panel, immunostaining of Sox9 following 5-HTP supplementation showed nuclear expression of the protein in interstitial and centro-acinar cells but not in acinar cells. Right panel: qPCR analysis showed comparable levels of Sox9 transcripts in control and 5-HTP-treated pancreata. **B**. qPCR analysis showed Hes-1 up-regulation and Ptf1a down-regulation in mouse pancreata following 5-HTP treatment. **C**. qPCR of Ptf1a and Hes1 expression in WT and TPH1$^{-/-}$ mice in the early phases after pancreatitis induction. Dexamethasone treatment of pancreatic acinar AR42J cells for 48 hours increased cellular content of amylase (**D**) and reduced Sox9 expression at both transcript (left panel) and protein (right panel) levels (**E**), indicative of increased cellular differentiation. **F**. qPCR analysis showed a time-dependent increase of Hes1 and decrease of Ptf1a expression in dexamethasone-treated cells. Results are average ± SEM (n=5), *p<0.05. Scale bars: 50 μm.

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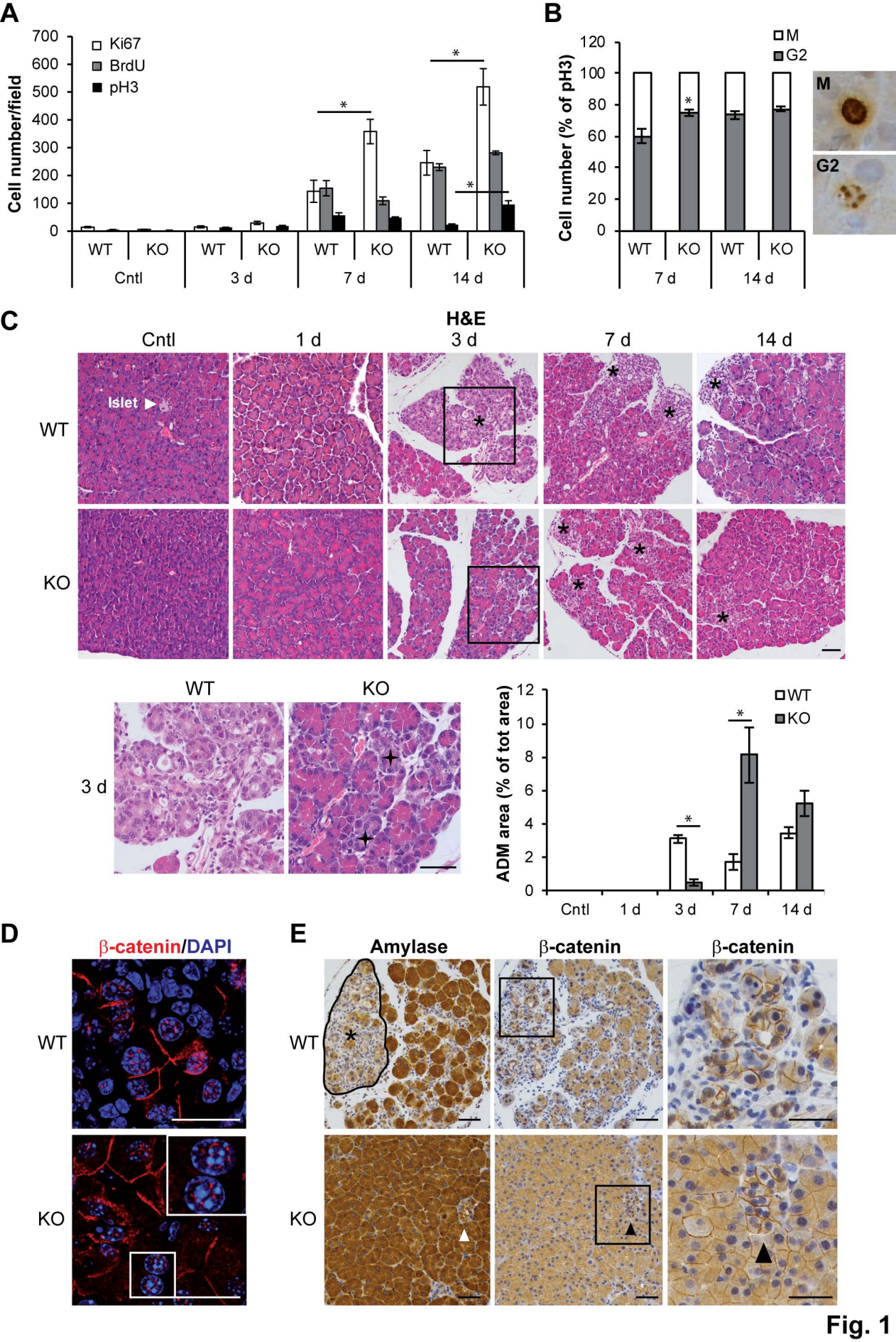
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BrdU
pH3
WT
KO
Cntl
3 d
7 d
14 d
B
Cell number (% of pH3)
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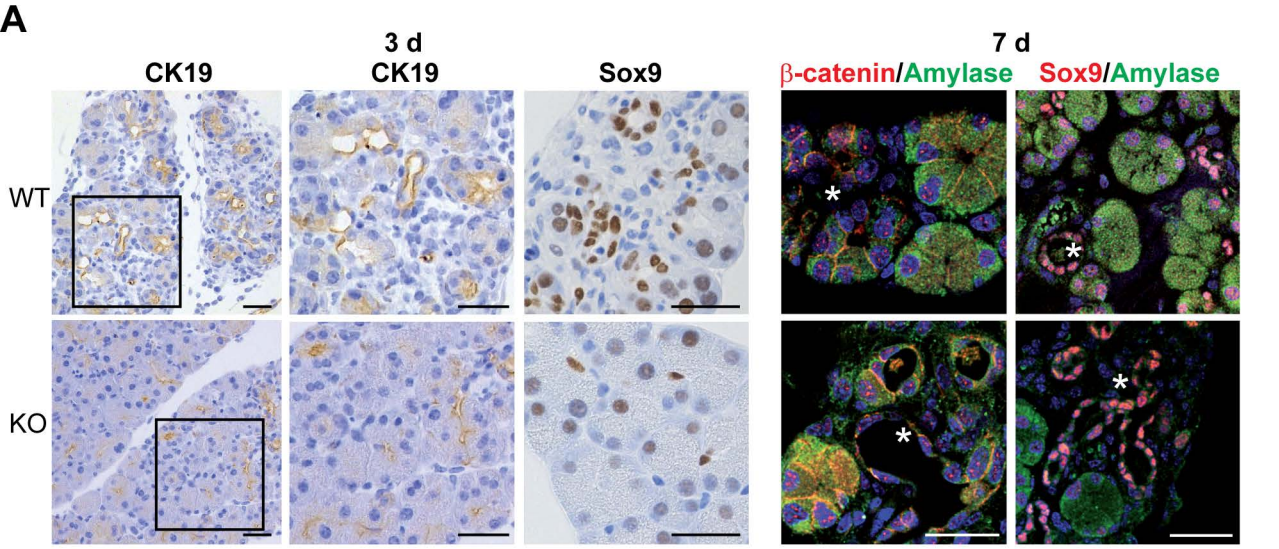
G2
C
H&E
Cntl
1 d
3 d
7 d
14 d
Islet
WT
KO
ADM area (% of tot area)
D
β-catenin/DAPI
E
Amylase
β-catenin

Fig. 1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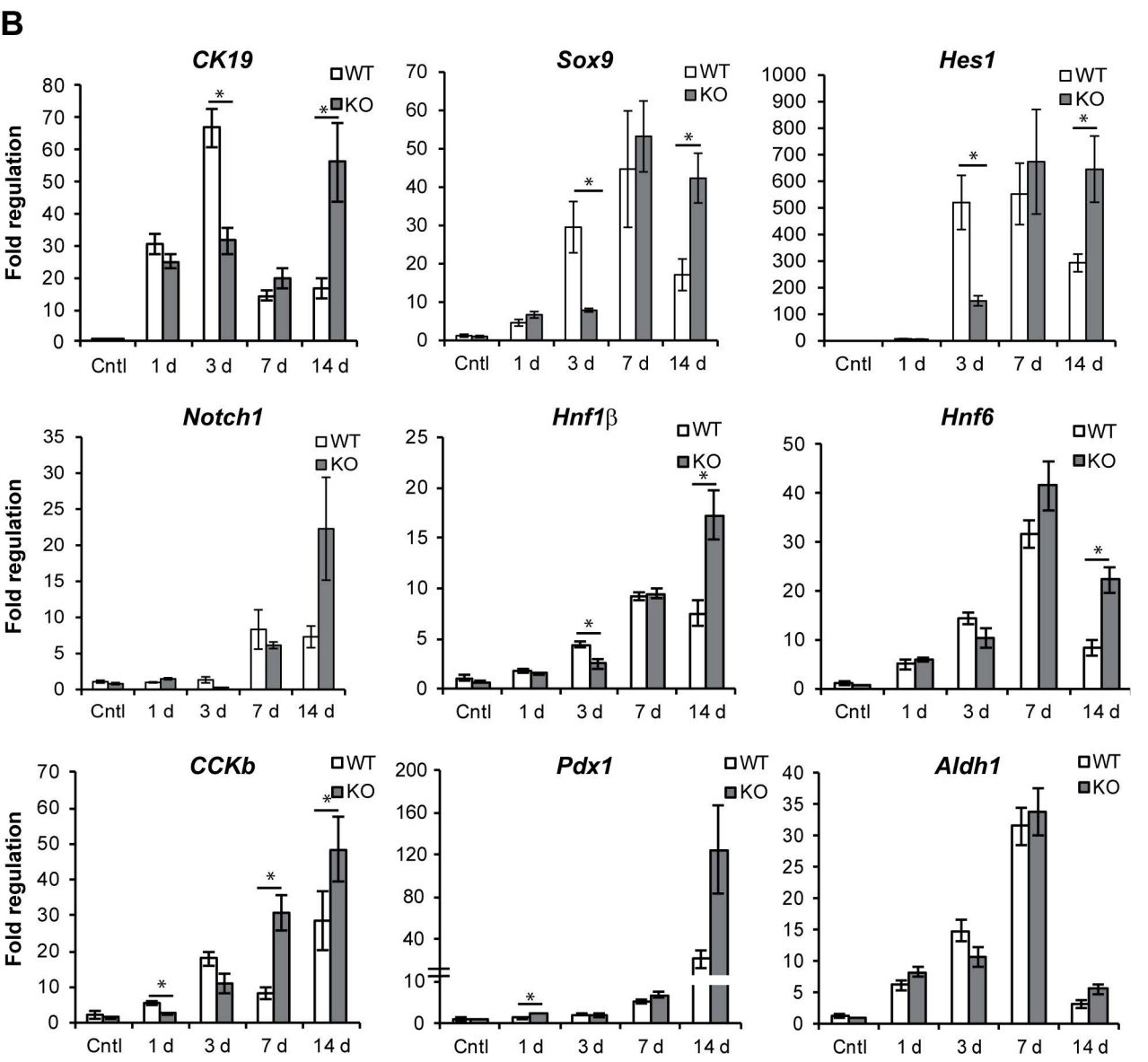
7 d
CK19
CK19
Sox9
β-catenin/Amylase
Sox9/Amylase
WT
KO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Sox9
Hes1
Notch1
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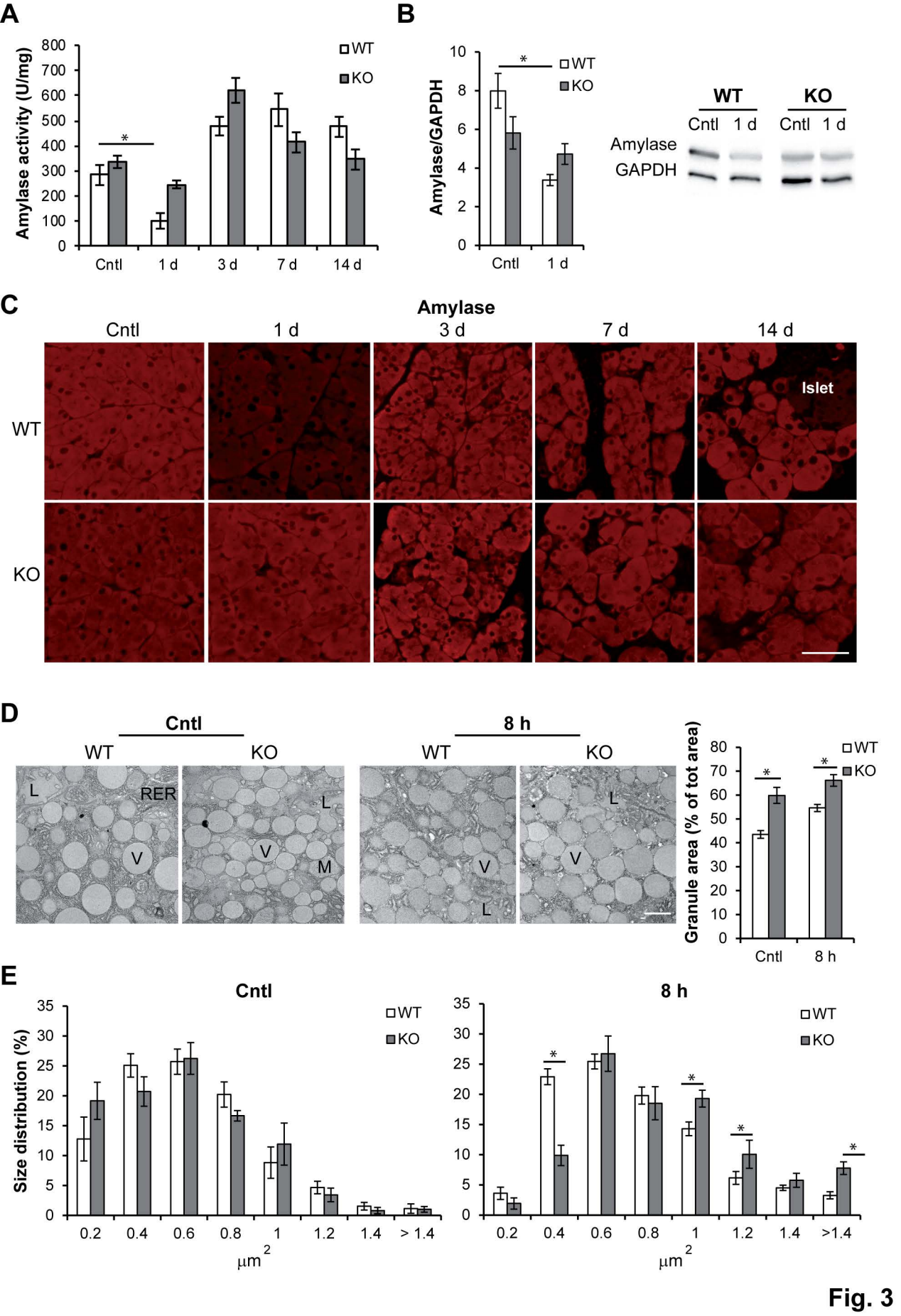
Aldh1
Fold regulation
WT
KO
Cntl
1 d
3 d
7 d
14 d

Fig. 2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700
600
500
400
300
200
100
0
WT
KO
*
Cntl
1 d
3 d
7 d
14 d
B
Amylase/GAPDH
10
8
6
4
2
0
*
WT
KO
Cntl
1 d
WT
KO
Cntl
1 d
Cntl
1 d
Amylase
GAPDH
C
Amylase
Cntl
1 d
3 d
7 d
14 d
WT
KO
Islet
D
Cntl
8 h
WT
KO
WT
KO
L
RER
V
L
V
M
V
L
L
V
V
L
Granule area (% of tot area)
80
70
60
50
40
30
20
10
0
*
*
WT
KO
Cntl
8 h
E
Cntl
8 h
Size distribution (%)
35
30
25
20
15
10
5
0
WT
KO
*
*
*
*
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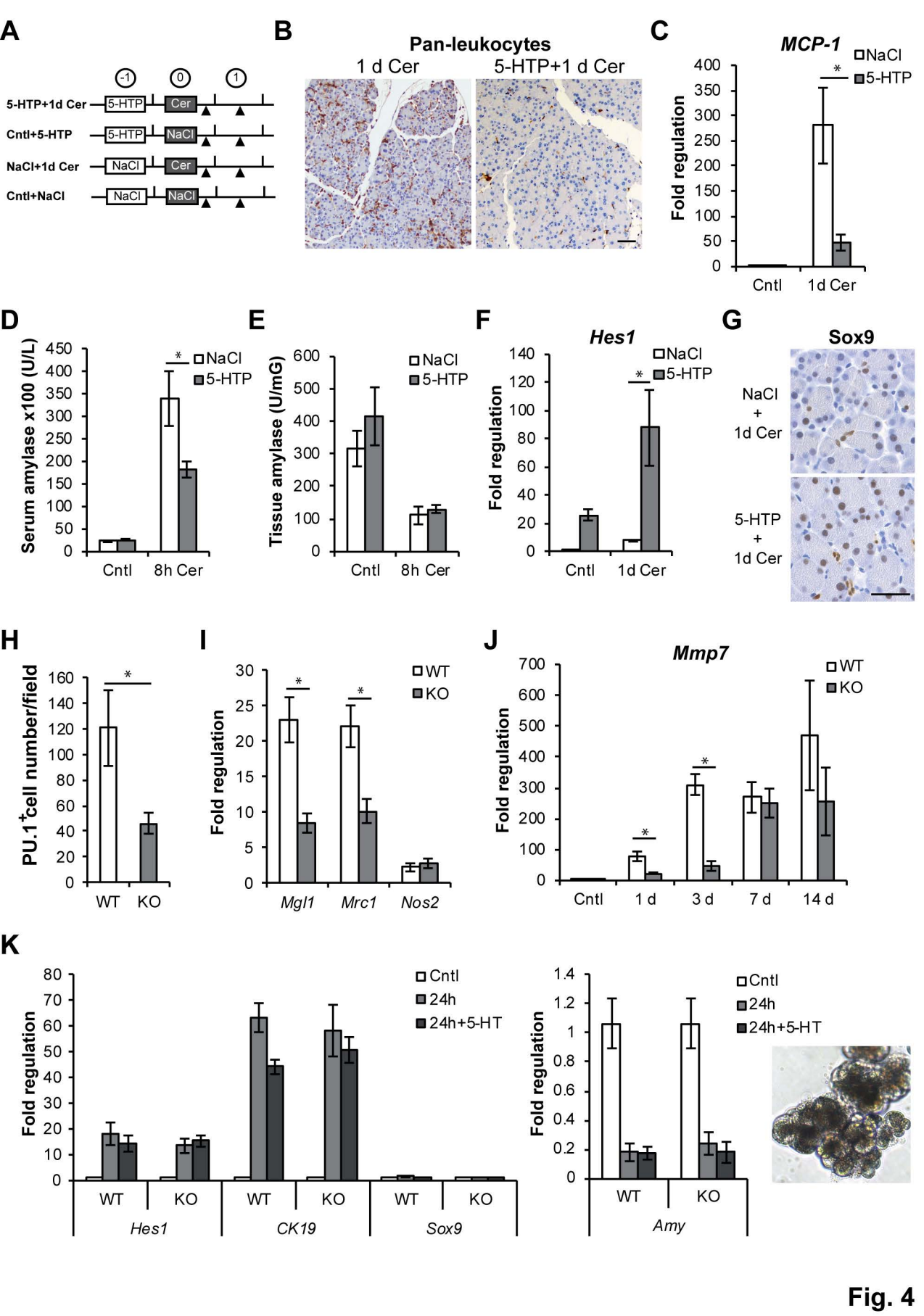
0.4
0.6
0.8
1
1.2
1.4
> 1.4
>1.4
μm 2

Fig. 3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NaCl
Cer
Cntl+NaCl
NaCl
NaCl
B
Pan-leukocytes
1 d Cer
5-HTP+1 d Cer
C
MCP-1
NaCl
5-HTP
Fold regulation
Cntl
1d Cer
D
Serum amylase x100 (U/L)
NaCl
5-HTP
Cntl
8h Cer
E
Tissue amylase (U/mG)
NaCl
5-HTP
Cntl
8h Cer
F
Hes1
NaCl
5-HTP
Fold regulation
Cntl
1d Cer
G
Sox9
NaCl + 1d Cer
5-HTP + 1d Cer
H
PU.1+cell number/field
WT
KO
I
WT
KO
Fold regulation
Mgl1
Mrc1
Nos2
J
Mmp7
WT
KO
Fold regulation
Cntl
1 d
3 d
7 d
14 d
K
Cntl
24h
24h+5-HT
Fold regulation
WT
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Sox9
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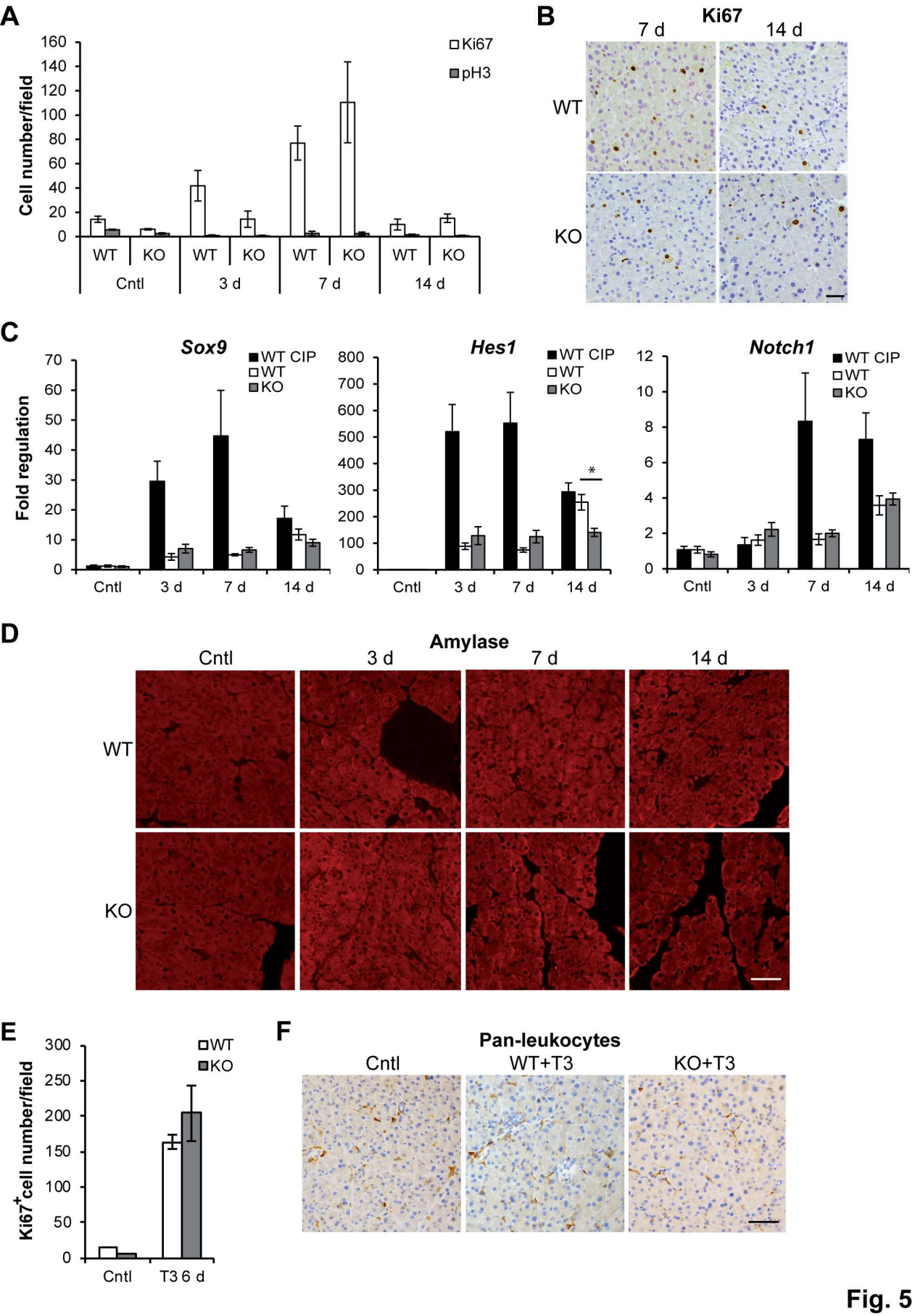
24h
24h+5-HT
Fold regulation
WT
KO
Amy

Fig. 4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7 d
14 d
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KO
C
Sox9
Hes1
Notch1
WT CIP
WT
KO
Fold regulation
*
D
Amylase
Cntl
3 d
7 d
14 d
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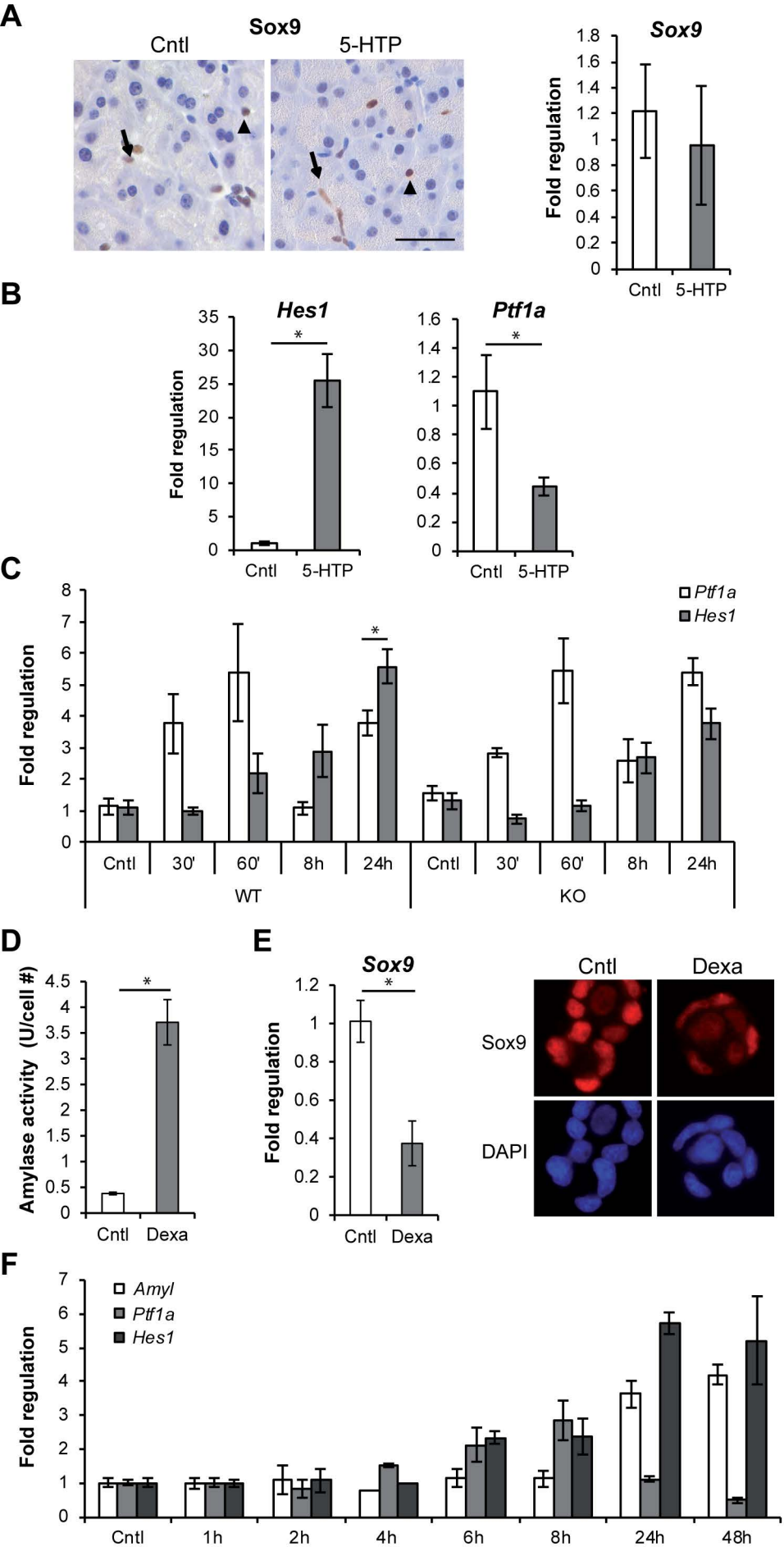
Ki67+cell number/field
T3 6 d
F
Pan-leukocytes
Cntl
WT+T3
KO+T3

Fig. 5


A
Sox9
Cntl
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Sox9
Fold regulation
Cntl
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B
Hes1
Fold regulation
Cntl
5-HTP
Ptf1a
Cntl
5-HTP
C
Ptf1a
Hes1
Fold regulation
Cntl
30'
60'
8h
24h
WT
KO
D
Amylase activity (U/cell #)
Cntl
Dexa
E
Sox9
Fold regulation
Cntl
Dexa
Cntl
Dexa
Sox9
DAPI
F
Amyl
Ptf1a
Hes1
Fold regulation
Cntl
1h
2h
4h
6h
8h
24h
48h

Fig. 6